

Comparative analysis of mucosal immunity to *Mycoplasma hyopneumoniae* in Jiangquhai porcine lean strain and DLY piglets

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ABSTRACT. The Jiangquhai porcine lean strain (JQHPL) is a new pork meat-type strain that has been developed in recent years from the parent lines Duroc, Fengjing, and Jiangquhai pigs (Duroc x Fengjing pig x Jiangquhai pig). Enzootic pneumonia (EP) in pigs induced by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is a chronic respiratory disease of pigs, generating high economic losses in the swine industry. Here, we investigated the degree of resistance to *M. hyopneumoniae* for the Jiangquhai porcine lean strain and the Duroc x Landrace x Yorkshire (DLY) pigs, which are Western commercial pigs that have been introduced in China. A total of 209 DLY piglets and 221 JQHPL piglets from 19 Landrace x Yorkshire and 22 JQHPL *M. hyopneumoniae* positive gestating sows with different expected dates of confinement were selected and raised in the same *M. hyopneumoniae*

positive farrowing barn. When the oldest suckling piglets were 37 days old, nasal swabs were collected from all the piglets (ranging from 4 to 37 days old) to detect the *M. hyopneumoniae* pathogen using n-PCR and *M. hyopneumoniae* specific SIgA using ELISA. Positive *M. hyopneumoniae* infection rates in both the strains increased with age; however, positive rates for JQHPL were lower compared to DLY at 14 to 35 days old. The level of the specific SIgA rose rapidly in JQHPL respiratory tracts, particularly in piglets 21 to 35 days in age compared to DLY piglets of the same age; however, the level of the specific SIgA in DLY also marginally increased. In conclusion, JQHPL pigs exhibits higher resistance to *M. hyopneumoniae* compared to DLY. It is possible that this characteristic is caused by the faster and stronger mucosal immunity phenotype of the JQHPL strain.

Key words: *Mycoplasma hyopneumoniae*; DLY; Resistance; Jiangquhai porcine lean strain; Mucosal immunity

INTRODUCTION

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia, which is a chronic respiratory disease in pigs. *M. hyopneumoniae* infections are highly prevalent in almost all swine producing areas, causing significant economic losses because of increased medication requirements and decreased performance of the pigs (Sibila et al., 2007, 2009; Maes et al., 2008). *M. hyopneumoniae* is transmitted by aerosolized droplets, and the crucial separation distance between herds for minimal infection risks is about 4.7 km (Dee et al., 2009). However, a new report described experimental aerosol movements of *M. hyopneumoniae* over distances of up to 9.2 km (Otake et al., 2010). It is generally accepted that the sow plays an important role in transmitting the *M. hyopneumoniae* to the offspring and, consequently, in maintaining the infection within the herd (Grosse Beilage et al., 2009). Moreover, inactivated vaccination does not significantly reduce the transmission of this respiratory pathogen (Meysn et al., 2006; Pieters et al., 2010; Villarreal et al., 2011; Chae, 2011). Therefore, the risk of spreading the disease to other groups is high, even if the groups are placed in separate pens.

At present, the control strategies for *M. hyopneumoniae* include management practices, optimal housing conditions, vaccination of piglets, and the administration of antibiotics (Grosse Beilage et al., 2009), based on the results of existing studies. However, reports remain sparse about whether different strains exhibit different levels of immunity to this disease. The Jiangquhai porcine lean strain (JQHPL) is a new meat-type strain that has recently been developed in China. It was developed using the parent lines Duroc, Fengjing, and Jiangquhai pigs (a Chinese native pig that is one of the most prolific pig strains in the world; Song et al., 2003). The breeding program including 5-6 generation selected after Duroc-Duroc-Jiangquhai pigs (DDJ) x Duroc-Fengjing-Jiangquhai pigs (DFJ) cross breeding, has been in progress for more than 10 years, resulting in a black pig breed. However, the inheritance of this phenotype and its correlation with disease susceptibility remain unknown. Therefore, this study investigated the mucosal immunity of JQHPL to *M. hyopneumoniae* after being naturally infected with the wild strain of *M. hyopneumoniae*.

MATERIAL AND METHODS

Selection of herds

The study was conducted on a pig farm located in Jiangsu Province, China. The sows from the two types of pig strains were kept on the farm: Landrace-Yorkshire and Jiangquhai porcine lean. To further ensure that the farm was a *M. hyopneumoniae* positive farm, two days before the experiment, *M. hyopneumoniae* in air was detected by Nested-PCR. At 8:30 to 10:00 am, a liquid cyclonic-filtration collector able to capture 18-20 L air/min was used to collect the air samples in the farrowing barn, nursery, and fattening house (with 30 min of operation in each location) as previously described (Dee et al., 2009; Pitkin et al., 2009), with some modifications. After centrifugation at 12,000 rpm for 30 min, the precipitation of each air sample was collected for n-PCR analysis.

Animals and experimental design

Nineteen Landrace-Yorkshire (LY) gestation sows and 22 Jiangquhai porcine lean (JQH-PL) gestation sows without clinical respiratory symptoms were housed in the same empty farrowing barn. The expected date of confinement (EDC) for all sows is presented in Table 1. None of the sows had received any type of *M. hyopneumoniae* vaccinations or *M. hyopneumoniae* sensitive antibiotics, and none of the sows had exhibited any signs of illness in the previous 6 months. However, all sows were confirmed to be *M. hyopneumoniae* positive after testing with an *M. hyopneumoniae* Antibody Test Kit (IDEXX Co, USA, data not shown). The breeding program of these sows was Duroc x LY and JQHPL x JQHPL; therefore, the new piglets born in the barn were Duroc x Landrace x Yorkshire (DLY) strain and JQHPL strains. When the suckling piglets were 7 days old, they were creep fed. During the experiment, none of the suckling piglets received any *M. hyopneumoniae* vaccinations or other immunologically active drugs active against *M. hyopneumoniae*, except leucomycin at a dose of 55 mg/kg, which was present in creep feed. The two types of sows were arranged at intervals in the farrowing barn, with no fostering occurring in this experiment. When the oldest piglets were 37 days, all of the piglets were divided into five groups (Table 2) according to their age. Nasal swabs were collected from all of the new piglets to detect the *M. hyopneumoniae* pathogen and specific anti-*M. hyopneumoniae* SIgA.

Clinical monitoring

The new suckling piglets were monitored daily at 8:30 to 9:30 am and 4:00 to 5:00 pm for clinical signs of *M. hyopneumoniae*, particularly coughing and dyspnea until the end of the experiment.

Nasal swab collection

The method of nasal sampling used in this study was previously described by Fablet et al. (2010) with some modifications. Both nasal cavities of each pig were swabbed with sterilized swabs that were inserted into the nostrils by rotation to reach deep into the turbinates. After the animal had sneezed 5 times the nasal swabs were removed from each cavity.

All samples were placed in 1.5 mL sterilized phosphate buffered saline (PBS) and incubated at 4°C overnight. After centrifugation at 12,000 rpm for 30 min at 4°C, the precipitate and supernatant were individually collected for n-PCR and ELISA.

Table 1. Number of the gestation sows.

Away from the EDC (day)	3-7	8-14	15-21	22-28	29-35
LY	3	4	4	5	3
JQHPL	4	5	4	6	3

EDC = Expected date of confinement. All the sows had not received any kind of *Mycoplasma hyopneumoniae* vaccinations or *M. hyopneumoniae* sensitivity antibiotic and exhibited no signs of illness in the previous six months, but were *M. hyopneumoniae* positive after testing with *M. hyopneumoniae* Antibody Test Kit. The two types of sows were intervals arranged in a farrowing barn. DLY = Duroc x landrace x Yorkshire; JQHPL = Jiangquhai porcine lean strain.

Table 2. Number of the new piglets.

Average age (in days)	7	14	21	28	35
Limit days of age	4-10	11-17	18-24	25-31	32-37
DLY	28	56	30	66	29
JQHPL	34	67	41	43	36

All the suckling piglets did not receive any *Mycoplasma hyopneumoniae* vaccinations or other immunologically active drugs active against *M. hyopneumoniae* except leucomycin in creep feed (55 mg/kg creep feed and feed from 7 days of age to the end of the experiment). Foster did not happen during the experiment.

DNA preparation

DNA samples from the precipitate were extracted using the TIANamp Bacteria DNA Kit (TIANGEN, China) following manufacturer protocols.

Nested-PCR for *M. hyopneumoniae* detection

A 427-bp fragment specific for the *M. hyopneumoniae* p P36 genome was amplified by the nested-PCR. The primer and cycle parameters are presented in Table 3. In brief, the outer primers PCR were assayed in 25 µL reaction mixture containing 2.5 µL 10X PCR buffer (Mg²⁺ free), 1.5 µL 25 mM MgCl₂, 2 µL dNTPs, 0.5 µL of each forward and reverse primer, 5 µL DNA, 0.2 µL 5 U/µL Taq, and 13.8 µL ddH₂O. After amplification, 1 µL undiluted PCR product was transferred to a new tube for the further amplification by the inner primers. The amplified products by the inner primers were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide (0.5 mg/mL).

Specific SIgA detection

The method of the indirect ELISA to detect anti-*M. hyopneumoniae* SIgA levels used in this study followed that previously described (Feng et al., 2010; Li et al., 2012), with minor modifications. In brief, polystyrene plates were coated overnight at 4°C with 5 µg per well of *M. hyopneumoniae* P97R1 protein diluted in carbonate-bicarbonate buffer, pH 9.6, then the plates were blocked with 1% bovine serum albumin (BSA) in PBS. After 3 PBS-T washes,

100 μ L BALFs were added to the plates in triplicate. Following incubation for 2 h at 37°C and 3 PBS-T washes, goat anti-pig IgA diluted at a ratio of 1:8000 in 1% BSA was added, and incubated at 37°C for 1 h. After 3 PBS-T washes, rabbit anti-goat IgG conjugated with horseradish peroxidase (1:10000 in 1% BSA) was added and incubated at 37°C for 1 h. After 3 PBS-T washes, the reactions were developed with 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide. OD readings at 450 nm were taken using a Dynatech MR 700 ELISA reader (Bectondickinson, USA) after 10 min.

Table 3. Primer and cycle parameters used for n-PCR.

Primer	Primer sequence (5'-3')	Fragment size/bp	Sequence accession	Annealing temperature (°C)	Cycles
Outer P ₁ Primers	5'-TTAGTGTCTCCCGT-TATG-3'	621	AY312243	42	34
P ₂	5'-GAAATCCGTATTCTCCTC-3'				
Inner P ₃ Primers	5'-TTACAGCGGGAAGACC-3'	427	AY312243	51	24
P ₄	5'-CGGCGAGAAACT-GGATA-3'				

RESULTS

Evidence of *M. hyopneumoniae* infection of the farm

To ensure that the farm was positively infected with *M. hyopneumoniae*, air samples in the farrowing barn that would be used in the experiment from the nursery and fattening houses nearby were collected and detected the *M. hyopneumoniae* pathogen. The result is presented in Figure 1. Based on the results of n-PCR that all the air samples from the five pigsties were *M. hyopneumoniae* positive indicating that the entire pigsty was infected with *M. hyopneumoniae*.

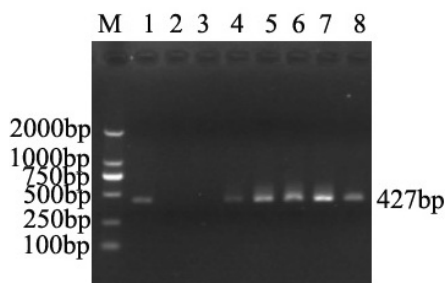


Figure 1. n-PCR analysis of the air samples in the farm. Lane M = Marker 2000; lane 1 = Positive control; lane 2 = Negative control (production of the first PCR as template); lane 3 = Negative control (ddH₂O as template); lanes 4 and 5 = Fattening houses; lanes 6 and 7 = Farrowing barn; lane 8 = Nursery house. These air samples are from five pigsties included the experimental farrowing barn and the nursery and fattening houses nearby in the farm. All the air samples from the five pigsties were *Mycoplasma hyopneumoniae* positive indicating that the entire pigsty was infected with *M. hyopneumoniae*.

Clinical observations

Most of the piglets showed good mental status and appetite. No clinical signs of pneumonia, such as cough and dyspnea were observed. Only two litters of DLY and two litters of

JQHPL were noted with diarrhea when they were 3 to 7 days old and 4 of the piglets died. There were no deaths from respiratory diseases.

Rate of positive samples by n-PCR

The number of positive samples and positive rates in DLY and JQHPL with different average ages are presented in Table 4. The general trend of the positive rate of *M. hyopneumoniae* infections in the two strain types increased as the animals became older. However, at 14, 21, 28, and 35 days of age, the positive infection rate of JQHPL was lower than that of DLY. The highest detection rate was found in DLY of 35 days old. In JQHPL, the highest detection rate was found in piglets that were 28 days old.

Table 4. Summary of positive rates detection by n-PCR.

Average age (in days)	7	14	21	28	35
DLY					
Number of positive samples	0/28	10/56	3/30	7/32	14/29
Rate of positive samples (%)	0	17.8	10	21.9	48.3
JQHPL					
Number of positive samples	2/34	6/67	6/41	8/43	4/36
Rate of positive samples (%)	0.59	8.96	14.63	18.6	11.11

For abbreviations, see legend to Table 1.

Local mucosal immunity

As shown in Figure 2, anti-*M. hyopneumoniae* SIgA secretion in the respiratory tract was measured in DLY and JQHPL on 7, 14, 21, 28, and 35 days old. The level of the specific SIgA in JQHPL respiratory tracts rose rapidly, especially from 21 to 35 days old when compared to the DLY, though the level of the specific SIgA in DLY also increased.

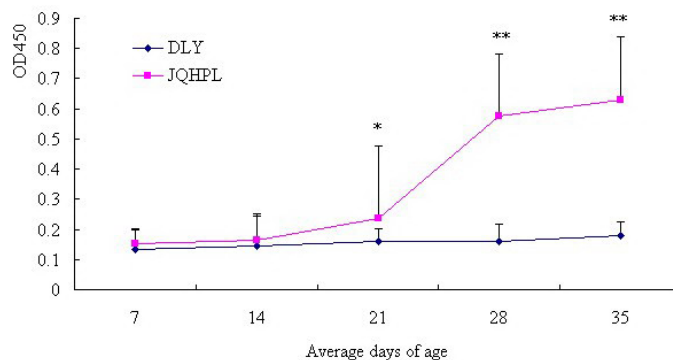


Figure 2. Detection of anti-*M. hyopneumoniae* SIgA in suspension of nasal swabs from the DLY piglets and JQHPL piglets of different age naturally infected in *M. hyopneumoniae*. Figures represented the ELISA analysis of *M. hyopneumoniae* specific SIgA. Although the concentration of SIgA were increased in both of the two type strains along the days of age, the relative concentration of SIgA in JQHPL was obviously higher compared to the DLY especially from 21 to 35 days old. Data are reported as means \pm SD. *Significant differences between the stressed group and the normal control group ($P < 0.05$); **Significant differences between the stressed group and the normal control group ($P < 0.01$).

DISCUSSION

Since the major routes of entry of *M. hyopneumoniae* is at the mucosal surfaces of the respiratory tract, the detection of this pathogen in the lower and upper respiratory tract could be used as an indicator that respiratory problems associated with MPS, which may start relatively early in the production system (Sibila et al., 2007). Nested PCR (n-PCR) of nasal swabs can be used to monitor the status of the disease at the herd level under field conditions (Otagiri et al., 2005; Nathues et al., 2012). Villarreal (2010) reported the occurrence and epidemiology of *M. hyopneumoniae* infections in pigs of different age groups in studies conducted from 1995 to 2009. To date, most detection methods used for suckling and weaned pigs is n-PCR of nasal swabs. The present study monitored the rate of natural transmission of *M. hyopneumoniae* in DLY and JQHPL piglets up to 37 days old reared in farrowing barns. Positive infection rates were lower for JQHPL piglets compared to DLY piglets from 14 to 35 days old. The results indicate that JQHPL pigs are more resistant to *M. hyopneumoniae* compared to DLY pigs.

The major route of *M. hyopneumoniae* entry is through mucosal surfaces of respiratory tract, with this pathogen spreading among the herd by airborne transmission. Mucosal immune system activation is the first signal of *M. hyopneumoniae* infection, but not humoral immunity (Herremans et al., 1999). Therefore, local humoral immunity appears to play an important role in this infection (Sarradell et al., 2003). SIgA is the major effector of mucosal immunity in the respiratory tract, which might form a protective membrane layer to eliminate invading pathogens and prevent active infection and colonization. SIgA detection may be used to monitor disease diagnosis (Cox et al., 2004; Parida et al., 2006). Our results showed an increase in the secretion of *M. hyopneumoniae* specific SIgA on the surface of aspiratory mucous in both types of piglets; however, the speed and strength of the secretion were higher in JQHPL piglets compared to DLY piglets, especially on 21 to 35 days. Therefore, JQHPL pigs have faster and stronger mucosal immunity compared to DLY pigs. This difference might explain why the positive infection rate was higher in 28-day-old JQHPL piglets compared to 35-day-old piglets. It is hypothesized that *M. hyopneumoniae* stimulated *M. hyopneumoniae* specific SIgA first, causing the concentrations of SIgA to increase, which probably neutralized *M. hyopneumoniae* to some extent. In comparison, the trend in DLY piglets was less pronounced; whereby, as the infection rate of *M. hyopneumoniae* increased, the concentration levels of SIgA only increased slowly. The results of this study indicate that mucosal immunity acts as an efficacious route to provide protection against *M. hyopneumoniae* infection.

CONCLUSIONS

The results of the present study demonstrated that the new meat-type strain of pig, named Jiangquhai porcine lean strain, is more resistant to *M. hyopneumoniae* infection compared to DLY piglets, which are the most widespread pig livestock used worldwide. The phenotype might, at least in part, contribute to the faster and stronger mucosal immunity of the Jiangquhai porcine lean strain. However, more information is required to elucidate the integrated responses that occur when intact *M. hyopneumoniae* infects the host. The acquisition of such information would contribute towards improving our understanding about the genetic and molecular mechanisms that regulate the pig immune response to *M. hyopneumoniae*, along with other similar types of pathogens.

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